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The Woodchuck: An Animal Model for Hepatitis B Virus Infection in Man

Key Words
Hepatitis B animal models
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Summary
Since the discovery of woodchuck hepatitis virus (WHV) in 1978, the virus and its host, the American woodchuck, have been studied and used as the most suitable model for human hepatitis B virus infection. WHV is closely related to the human virus, having strong similarities in morphology, genome structure and gene products, replication, epidemiology, the course of infection and in the development of illness and hepatocellular carcinoma. Because of this high homology, the woodchuck model is used for many studies for the development of new vaccines, therapeutic vaccination and antiviral agents. In addition, the woodchuck system is used for investigation of molecular mechanisms of the viral life cycle, the mechanisms of carcinogenesis and cell infection.

Introduction

A high frequency of primary hepatocellular carcinomas (HCC) in woodchucks *(Marmota monax)* kept in the Philadelphia Zoo led to the identification of the woodchuck hepatitis virus (WHV) as the first animal hepatitis B-like virus [1, 2]. HCC is the major cause of death in these animals followed by heart attack due to atherosclerosis. For some time woodchucks have been used as a model for atherosclerosis in man [3, 4]. Further studies at the Penrose Research Laboratory associated to the Philadelphia Zoo and at the NIH, Washington, established the close association of chronic WHV infection and development of HCC [5–7].

Prevalence of chronic WHV infection varies widely between the states of the East Coast in North America. WHV was shown to be endemic in the eastern woodchuck in the mid-Atlantic states of the USA. In Delaware and Maryland, up to 30% of woodchucks are chronic carriers, whereas in the State of New York and New England, in general, carriers are not observed. The European marmot *(Marmota marmota)* does not seem to be susceptible to WHV [8].

Although the chimpanzee has long served as a surrogate host for humans in modelling hepatitis B virus (HBV) infection, the chronic disease in this model is less severe and hepatocellular carcinoma has not been observed [9, 10]. The woodchuck is currently used in many laboratories to study pathogenesis of hepadnavirus infection, molecular mechanisms of HCC development, and cell tropism of hepadnaviruses. Woodchucks are also used to study different approaches for new vaccines to hepadnaviruses and evaluation of antiviral drugs in chronic WHV infection. Woodchucks are also a highly sensitive model to test viral inactivation procedures.

Ponzetto et al. [11] transmitted hepatitis delta virus (HDV) to woodchucks which are chronic carriers of WHV and established the woodchuck as an in vivo model for

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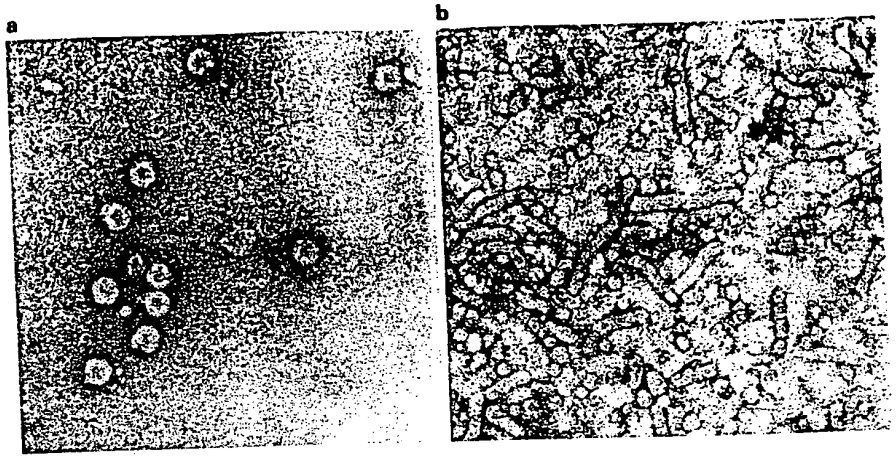

Fig. 1. Complete WHV virion (**a**), spherical and filamentous particles (**b**) of WHs antigen.

HDV replication studies [12–14]. Recently, two modes of HDV infection, simultaneous HDV/WHV infection in woodchuck without any evidence of prior exposure to WHV and superinfection of chronic WHV carriers, have been established [15].

The association of chronic WHV infection and HCC, including molecular mechanisms of viral integration in the host genome, was reviewed recently [16]. The present paper deals with the woodchuck as a model for the natural course of hepatitis B, vaccine development and an in vivo model for antiviral therapy.

Morphology of WHV

The morphology of WHV is very similar to HBV [1]. Mature virions form spherical enveloped particles 45 nm in diameter for WHV, 42 nm for HBV. The viral envelope consists of all three virus-encoded surface antigens (S-WHs, M-WHs, L-WHs) and host cell-derived lipids; the inner shell consists of the nucleocapsid (core) with a diameter of 27 nm. The nucleocapsid contains the genome, consisting of a partially double-stranded DNA genome, noncovalently linked to form an open circle by partial overlap and a viral polymerase that can fill up the single-stranded region [17, 18]. The priming of the minus strand synthesis by the terminal protein encoded by the *P* ORF (TP domain) has been studied in detail [19]. Beside mature virions, empty particles consisting of surface antigens and host cell-derived lipids are released in abundance into the plasma of infected animals. The empty particles have a typical diameter of 20 nm and are spherical or tubular, sometimes forming long filaments (fig. 1).

Genome Structure

The WHV genome size of 3,300 nucleotides is somewhat larger than the HBV genome of about 3,200 nucleotides. Hepadnaviruses are among the smallest known animal viruses, therefore their genome is very compact and efficiently organized (fig. 2). Five isolates of WHV were originally cloned and their nucleotide sequences determined [20–23]. Their genomic organization is identical and they are as well conserved as the HBV genotype encoding HBsAg subtype adr [24]. The sequence heterogeneity seems to be low, but defective WHV genomes were found in the sera of chronic carriers. There is no evidence for a requisite role of defective particles in the establishment of persistent hepadnavirus infection [25].

Four partially overlapping open reading frames can be deduced from the DNA minus strand nucleotide sequence: the *C* ORF, coding for the major nucleocapsid, or core antigen and a precore protein which is cotranslationally processed and secreted by hepatocytes as WHe antigen; the *S* ORF coding for the large (preS1), middle (preS2), and small surface antigen (s-Ag); the *P* ORF, encoding for the viral reverse transcriptase, RNAse H and the genome-linked protein, and the *X* ORF, which codes for a protein that has transactivating properties. Different proteins are derived from the same open reading frame by generation of RNA transcripts with heterogenous 5′-ends that use downstream AUG start sites. Computer-based alignments of amino acid sequences from viral proteins demonstrate that WHV shows a high homology to HBV (table 1).

Transcripts of WHV

Three major polyadenylated, unspliced transcripts with molecular sizes of 3.5, 2.4 and 2.1 kb have been iden-

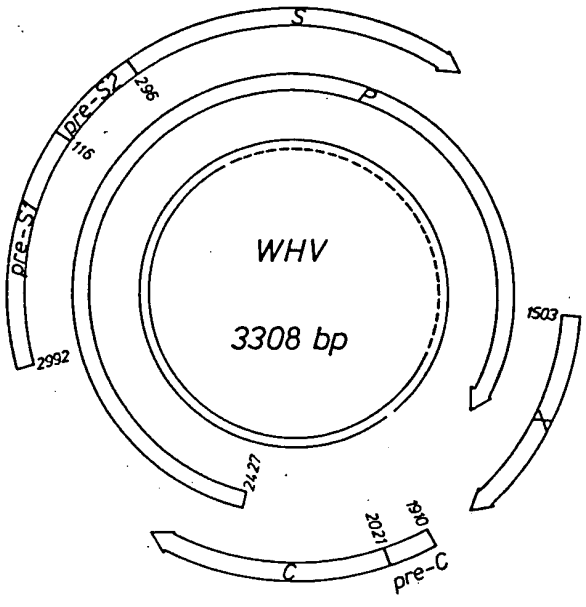

Fig. 2. Organization of the WHV genome.

Table 1. Comparison of *C* gene, *S* gene, and *X* gene of HBVadr1 and WHV1

ORF	% homology nucleic acid	% homology amino acid
C gene	70.1	75.2
S gene	73.2	60.2
X gene	65.7	47.8

tified for HBV [26–29] and two of 3.7 and 2.1 kb for WHV [30, 31]. The minor transcript of 2.4 kb encoding the preS1 ORF has been described in HBV-infected chimpanzee liver [32, 33], but no corresponding RNA has been detected in WHV-infected livers using hybridization techniques [30, 31]. The competence of the pregenomic RNA to direct viral DNA synthesis has indirectly been validated by experiments in which the cDNA equivalent of pregenomic WHV RNA was fused to heterologous promoters. Transfection of such constructs into cultured cells leads to the propagation of infectious virus particles. Using these constructs, expression of hepadnaviruses is no longer restricted to hepatoma cells. The expression of WHV in fibroblasts leads to the production of infectious virus as determined by the inoculation of susceptible woodchucks [34]. From these experiments it can be inferred that the steps in the hepadnaviral replication cycle that follow the synthesis of pregenomic RNA do not depend on hepatocyte-specific factors [34].

Gene Products of WHV

The nucleocapsid antigens are encoded by the *C* ORF and are highly conserved in HBV and WHV (66%) [24]. The core antigen is a basic protein of 21.5 kD, possessing a nonspecific DNA binding capacity. In the ORF of WHV core there are two adjacent ATGs, and it is unknown which of these two is used for the translation start for the precore protein. In HBV there is only one ATG at this position. The precore protein contains a signal sequence that is cotranslationally processed [35]. The presence or absence of the amino-terminal precore sequence influences the subcellular distribution of the core proteins [36, 37]. Proteins lacking the precore sequence accumulate in the form of core particles in the cytoplasm of WHV- infected cells, but they are found in the nucleus of HBV-infected cells [36]. The reason for this different localization is not known. Polypeptides containing the precore sequence can be proteolytically processed and secreted in the serum, where they appear as e antigen.

The WHe antigen is an N-glycosylated protein with a molecular weight of 24 kD [38]. It is dispensable for viral replication in the woodchuck but may be important for the establishment of chronic infection [39].

Core-specific proteins that are consistent in size with those having a translation start at the precore AUGs could be demonstrated in WHV-infected livers in processed as well as in unprocessed form [34]. The major core antigen found in WHV-infected livers corresponds in size to the 21.5-kD protein as translated from the second AUG (core AUG) in vitro.

Within the *S* ORF are three AUGs which are used to produce preS1 antigen (L-WHs), preS2 antigen (M-WHs) and S antigen (S-WHs) from one ORF. The molecular sizes of the proteins in a glycosylated (gp) and unglycosylated (p) form have been determined: WHs L protein p45 and gp47, M protein p33 and gp36, S protein p23 and gp27 [40, 41].

The major surface protein species identified in all hepadnavirus infections is the small WHs protein translated from the *S*-mRNA. The 20-nm subviral particles consist mainly of small surface proteins. The filaments also contain preS proteins. The S antigens are cross-reactive between WHV and HBV [42]. WHsAg can elicit neutralizing antibodies to HBV in chimpanzees [43]. HBV

preS2 binds polymerized human serum albumin [44], but this type of binding could not be identified for WHV preS [45].

X Protein

The function of the *x* gene during viral infection is not yet known. A deletion mutant with a translational stop in the *X* ORF near the beginning of the last third of the coding sequence, which replicates normally in a transient transfection assay in hepatoma cells, shows that an intact *X* product is dispensable for HBV replication [46]. WHV *X* protein-deficient mutants were viable for WHV DNA replication in vitro, but the levels of viral DNA were reduced in infected cells. These mutants, however, did not initiate a detectable infection in susceptible woodchucks [47, 48]. Therefore the *X* protein seems to be essential for WHV infection in the host.

Natural and Experimental Infection of Woodchucks

The natural route of WHV infection in the woodchuck is thought to be the same as for HBV, by blood and secretions. Natural infection of woodchucks seems to occur in utero or at birth. In the wild, woodchucks are born after hibernation in March and April and viremia is observed 2–3 months later. 30% of the animals develop a chronic carrier state. In the early 1980s, the NIH funded the establishment of a woodchuck breeding colony at Cornell University in order to pursue investigations on the role of hepadnaviruses in liver disease. Since then, at Georgetown University and the National Institute of Allergy and Infectious Diseases (NIAID), research has been conducted to develop the woodchuck as an experimental animal model to define the natural history of hepadnavirus infections and liver disease under controlled laboratory conditions [7]. Experimental inoculation of colony-bred, neonatal woodchucks with standardized virus challenge pools results in uniform kinetics of WHV infection and predictably high rates of chronicity (60–70%) [49].

The rate of chronicity after experimental inoculation of newborn animals, i.e. within a week of birth, is high and is dependent upon the inoculum used [7, 50]. In contrast, inoculation of juvenile or of adult animals (2 months or older) with different inocula caused acute infection and resulted in a carrier rate of 15%, independent of the inoculum used. It is unknown whether the difference in the rate of chronicity using different strains is due to minor differences in infectious dose or genetic differences between WHV strains. The mode of transmission is very similar to the HBV infection of man. Perinatal infection results in a carrier rate of 95% in babies, whereas infection of adults results in a carrier rate of about 5–10%.

The incubation period for WHV infection in the woodchuck is about 8–14 weeks, depending on the infectious dose. In experimental infections the first marker of viral replication is the appearance of WHV DNA at about 6–8 weeks after inoculation. Surface antigen (WHsAg) and antibodies to the core protein (anti-WHc) are detected about 4 weeks later [51] (fig. 3). WHV core protein can be detected in hepatocytes 6 weeks after inoculation [52].

The involvement of different tissues in the replication of WHV has been studied in detail and seems to be very complex [49, 53, 54]. The first appearance of cellular WHV DNA occurs in lymphoid cells of the bone marrow, although no evidence of viral replication was observed there. Active replication of WHV first occurs in the liver followed by active replication in spleen, lymphocytes and lymph nodes. During that period, WHV disappears from the bone marrow. In a late stage, nonreplicating WHV DNA appears in the lymphoid cells of the thymus. It is possible that the presence of WHV DNA in thymus and bone marrow during recovery of woodchucks is related to the complex response of the immune system to acute WHV infection superimposed upon the normal development of the immune system. In summary, these studies indicate that the lymphoid system is intimately involved in the natural history of WHV infection from the earliest stage of virus entry.

Additional studies have shown that lymphoid cells in the spleen and peripheral blood lymphocytes (PBLs) in the woodchuck may actively replicate WHV, or at least contain nonreplicating WHV DNA [55, 56]. PBLs from chronic carrier woodchucks containing nonreplicating WHV DNA can be activated upon mitogen (lipopolysaccharide)-induced proliferation of PBLs [57]. These stimulated PBLs release complete virions, which are infectious after inoculation in WHV-negative woodchucks [58]. The role of PBLs containing latently replication-competent WHV for the maintenance of the chronic carrier state is unknown.

WHV DNA was found to persist in a nonreplicating state in the hepatocytes of convalescent woodchucks several months after seroconversion to anti-WHs. Up to 25% of woodchucks that serologically converted to anti-WHs were found to carry low levels of WHV DNA in hepatocytes and lymphocytes. These observations indicate that some woodchucks may never clear WHV genomes after acute infection. Persisting WHV genomes in hepatocytes may be the reason that even anti-WHs-positive woodchucks show a higher risk (17%) of developing HCC than WHV-negative animals in which HCC is not found [7].

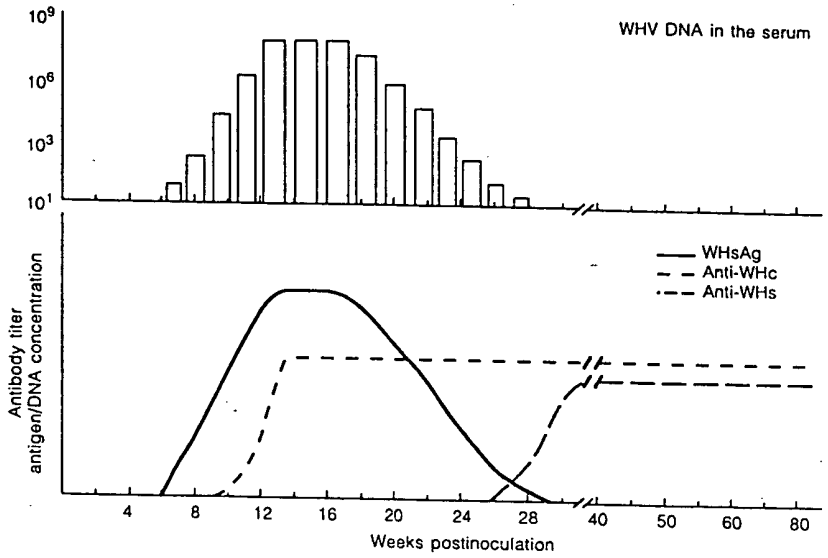

Fig. 3. Schematic pattern of markers following experimental infection of woodchuck with WHV [modified from Korba et al.].

The mechanism by which WHV ultimately is eliminated in transient infection of the liver is not yet understood. Recent experiments in the duck and woodchuck have shown that clearance of the virus which has been present in virtually all hepatocytes occurs after less than 4 weeks in woodchucks and is event faster in the duck [59, 60]. The contribution of the cellular immune system to cell death is not known. Kajino et al. [60] propose different models of how viral elimination may occur:

(1) Infected hepatocytes are destroyed by the immune system and are replaced by division of a pool of uninfected progenitor cells. If most of the infected cells were destroyed simultaneously by the immune response, fulminant hepatitis would occur. However, fulminant hepatitis is a rare event in man and woodchuck.

(2) Hepatocytes that are successively destroyed by the immune response are replaced by division of adjacent mature hepatocytes, which may or may not be infected. Viral DNA in infected hepatocytes is distributed to daughter cells, but virus replication is inhibited once hepatocytes enter the cell cycle, so that virus is eventually lost by 'dilution' as cells undergo multiple rounds of division. There is evidence of an increased turnover of hepatocytes and inhibition of replication in cells that enter the cell cycle.

(3) Virus is spontaneously cleared from individual, nondividing hepatocytes. It is possible that cytokines released by cytotoxic T lymphocytes (CTL) directly inhibit viral DNA replication or transcription as has been described in mice that are transgenic for HBV [61].

Persistent Infection and HCC

Persistent WHV infection is generally associated with chronic hepatitis of varying severity [6, 62, 63]. It is characterized by mild to severe portal or periportal inflammation with occasional necrosis, bile duct proliferation and appearance of ground glass cells resembling the healthy carrier state in humans [62]. Foci of altered hepatocytes resembling those found during the course of chemical carcinogenesis might represent preneoplastic nodules [64]. Woodchucks chronically infected with WHV at birth almost inevitably develop HCC [7]. Taking the shorter lifespan of woodchucks into account, HCCs in woodchucks occur much more frequently in WHV-infected woodchucks than in HBV-infected humans. The incidence of liver tumors in WHV-infected woodchucks is the highest, second only to that following carcinogenic treatment, suggesting that WHV is one of the most efficient oncogenic agents among known hepatocarcinogens [16]. WHV has also the highest oncogenic potential among the mammalian hepadnaviruses. A comparison of HCC development in woodchucks infected with WHV versus ground squirrel hepatitis virus (GSHV) showed that HCC arises rapidly and more frequently in WHV infection indicating that viral determinants which are thus far unknown, rather than host specificities, seem to be re-

sponsible for the different oncogenic potential of mammalian hepadnaviruses [65]. Aflatoxin B_1 (AFB_1) seems to be much less hepatocarcinogenic for woodchucks than WHV [66]. In contrast to previous findings, recent results by Bannasch et al. [unpubl. results] show that AFB_1 treatment accelerates HCC development and alters the frequency of HCCs in chronically infected woodchucks.

The molecular mechanism by which chronic WHV infection generates HCC in woodchuck has been reviewed recently by Buendia et al. [16]. Southern blot analysis of genomic DNA from a large number of woodchuck HCCs provide evidence of integrated WHV sequences in about 90% of tumors, both in chronic carriers and tumors of woodchucks which seroconverted. WHV, in contrast to HBV, seems to activate cellular proto-oncogenes by integration of viral sequences in the flanking host DNA [67]. In the efforts to identify such insertions of WHV, Buendia and co-workers [68–73] found a high frequency (50%) of insertions of WHV DNA adjacent to C-myc and N-myc sequences. The N-myc messenger RNA was found to be overexpressed. The activation of N-myc expression in woodchuck HCC is similar to that caused by the murine leukemia virus (MuLV). In both cases, insertion of virus sequences occurs in the 3′ noncoding region of the N-myc locus in the same transcriptional orientation leading to chimeric RNA. Recent results by Fourel et al. [73] have demonstrated that HCC of woodchucks which have no integration of WHV DNA sequences close to N-myc nevertheless show integration of WHV DNA 20 kb away on the same chromosome.

The Woodchuck as a Model for Vaccine Development

Vaccination with WHsAg

Immunization with purified serum-derived or recombinant surface antigen of hepatitis B virus (HBsAg) protects against virus infection, and forms the basis of the currently available, efficient and safe vaccine against HBV infection. However, recipients of vaccine need boosters to maintain a sufficient antibody titer, some nonresponders occur, and recently, escape mutants have been observed. A vaccine of serum-derived WHV surface antigen prepared in a similar way from plasma of chronic carrier animals is also protective in woodchucks [74]. Synthetic peptides of the HBsAg encompassing aa 110–135 and 125–137 have been evaluated as vaccines in the chimpanzee model [75]. Two out of 9 animals were completely protected and 6 out of 9 animals revealed pattern of at least partial protection. Synthetic peptides to the corresponding sequence of WHs protein have been evaluated. Two of 3 woodchucks immunized with the peptide spanning aa 110–135 of WHs developed anti-WHs and were completely protected. This preliminary analysis indicated that region 110–135 of the *S* gene carries protection-inducing epitopes of both HBsAg and WHsAg.

Subsequent vaccination studies were performed to determine whether WHsAg and HBsAg show epitopes which elicit protective immunity [43]. In these cross-vaccination studies chimpanzees were immunized with WHsAg and woodchucks with HBsAg. Immunization of chimpanzees with WHsAg protected these animals against HBV challenge indicating the presence of a cross-reacting epitope in WHsAg. The degree of protection obtained by cross-vaccination appeared to be related to serological levels of cross-reactive antibodies to HBsAg present at the time of HBV challenge in the chimpanzee. In contrast, woodchucks vaccinated with HBsAg developed only HBsAg-specific antibodies and were not protected from WHV infection. The humoral anti-HBs response did not alter the course of WHV infection and disease which was consistent with a lack of cross-reacting antibody response (anti-WHs). Apparently cross-vaccination had no priming effect against WHV in the woodchucks. These results suggest that the lack of cross-reacting anti-WHs in the woodchuck was due to the lack of humoral response to the neutralizing epitope determinants of HBsAg. These cross-vaccination studies indicate the presence of at least one shared epitope that stimulates antibodies in chimpanzees which is associated with protection from HBV infection.

Vaccination with Core Protein of HBV and WHV

In recent years the immune response to proteins of the nucleocapsid of different viruses has been studied in detail. CTL against nucleoproteins have been identified, e.g. those of influenza A [76], human immunodeficiency virus [77], rabies virus [78], and lymphocytic choriomeningitis virus (LCMV) [79]. It is believed that they are important in eliminating virus after infection.

Immunization with HBcAg partially protects chimpanzees from HBV infection [80–82] and immunization with WHcAg completely protects woodchucks against infection after challenge with WHV [83, 84]. The mechanism of protection after immunization by an internal virus component such as HBcAg or WHcAg remains unknown. Neither protein can be recognized by virus-neutralizing antibodies. Many other examples of protective immunity induced by internal viral components are

due to the induction of CD8+ CTL mainly by live recombinant viruses that deliver the recombinant antigens to the MHC class I pathway [85]. In vivo induction of MHC class I restricted CD8+ cytolytic T cells has recently also been described after immunization with lipopeptide [86], with ISCOM particles containing a synthetic peptide, and with free synthetic peptide in complete Freund's adjuvant (CFA) [87–89]. CTL in HBcAg-immunized chimpanzees and in WHcAg-immunized woodchucks have not yet been investigated due to technical problems [57]. Therefore it can only be speculated that protection after immunization with core protein is mediated by core-specific T-cell help for antibody production against surface structures [90, 91] and/or by help for, or induction of CTL, which would require infection and replication of WHV. However, in the studies by Roos et al. [83] and Schödel et al. [84] no markers of viral replication in sera or liver of protected animals after challenge were seen. This may be due to a lack of sensitivity of the assays used. If intrastructural/intermolecular help by T cells against WHcAg for antibody production were to account for protection, a secondary response to WHsAg would be expected in woodchucks immunized with WHcAg after challenge. The anti-WHs response developed indeed faster in WHcAg-immunized animals protected against WHV challenge than in unprotected immunized or nonimmunized animals. In the latter groups, anti-WHs was only detected after the appearance of circulating WHsAg and overlapped with clearance of WHV. The data demonstrate that intermolecular/intrastructural T-cell help for the production of anti-surface antibodies is operative in this replicative model. However, they do not prove that the early induction of virus-neutralizing antibodies is the role or major mechanism responsible for the protection observed.

In further studies, immunization was performed against WHV infection in the woodchuck [84]. After two immunizations with 50 µg HBcAg, 2 of 6 woodchucks were completely protected from infection with WHV (10^6 ID_{50}). The protection from WHV infection in 2 of 6 woodchucks immunized with HBcAg is probably due to the homology that is shared between the amino acid sequence of the core proteins of HBV and WHV. These experiments were the first step to investigate the minimal amino acid sequence homology of the core protein that is capable of protecting woodchucks against WHV infection. As a matter of fact, several regions of more than 10 amino acids are conserved in both proteins. Two of these regions of identity, aa 100–110 and 120–140, have been reported to be major T-cell epitopes of HBcAg, at least in the mouse system [91]. Further immunizations with HBcAg and conserved peptides which have been shown to be T-cell epitopes [92] in a larger number of animals, with well-defined inocula for the challenge, are necessary to define more precisely this partial protection.

Vaccination by Recombinant Core Particles Expressed by Live Attenuated Salmonellae

Expression of recombinant core particles containing immunodominant epitopes of the surface protein [93, 94] in live attenuated salmonellae could form the basis of an oral route vaccine against HBV in man. In a pilot study, 3 animals were immunized with a high dose of recombinant *Salmonella typhimurium* that expressed the authentic WHc protein (X4064, pWc). One of 3 woodchucks reacted with a detectable anti-WHc titer and was protected from subsequent challenge [84]. The low anti-WHc response is probably due to the inability of salmonella to grow in woodchuck tissues. This was not completely unexpected as salmonellae expressing *Escherichia coli* heat-labile enterotoxin B (LT-B) fusion proteins when fed to woodchucks failed to induce detectable anti-LT-B responses. At the same tissue LT-B proved to be a potent immunogen delivered by this route to mice. Woodchucks also tolerate the oral administration of $>10^8$ CFU of virulent *Salmonella enteritidis* and *S. typhimurium* strains, showing no signs of illness and reacting with low serum antilipoprotein titers. Until salmonellae are identified that productively infect woodchucks it will be impossible to test attenuated salmonellae as carriers of WHV antigens, e.g. WHsAg, WHcAg or recombinant WHV core particle which carry preS or S epitopes [93, 94] in this species.

Vaccination against Hepatitis D Virus Infection

Based on the protection against WHV infection by recombinant core proteins, several studies have been performed to protect woodchucks from HDV superinfection by immunization with liver-derived [95] and recombinant HDV protein [96]. Ponzetto et al. [96] immunized chronic WHV carrier woodchucks with a recombinant, complete, small HD protein (195 aa). In 2 of 4 animals after WHV challenge, HDV RNA failed to appear and in two other vaccinees viremia was mild and transient in comparison to control infections. HDV antigenemia was either absent or lower in the four vaccinees than in control animals. Immunization protocols with vaccinia recombinants expressing the larger HDV open reading frame 5 and recombinant HD proteins expressed by the baculovirus system did not elicit a humoral immune response to HDAg and did not prevent superinfection with HDV.

However, they reduced the level of viremia in immunized animals [P. Karayiannis et al., unpubl. results]. These preliminary results indicate that vaccination of human HBV carriers with HDAg may be a realistic objective in order to minimize superinfection with HDV. Further studies, however, will be needed to improve the immune response to HDV proteins.

Therapeutic Vaccination of Chronic WHV Carrier Woodchucks

Vaccines have been commonly used to prevent infection and disease. Some recent studies suggest that vaccines cannot only prevent illness but are also useful for treatment of chronic infections. This idea has received much public attention from efforts to boost the immune system, e.g. of people infected with HIV [97], with herpes simplex virus [98], HBV [99], or *Mycobacterium leprae* [100]. The first study with HBV was published by Pol et al. [99] showing the potential efficacy of immunotherapy for the control of HBV replication in HBV chronic carriers. They treated 14 patients who were chronically infected with HBV using the current HBsAg vaccine. After 6 months, HBV DNA could no longer be detected in 3 patients. Another 4 had detectable but significantly decreased amounts of HBV DNA. In comparison, among a historical control group of 34 patients who were followed over 40 months, only 3 spontaneously eliminated HBV. During HBV infection, immune response against various HBV proteins is stimulated. In acute, self-limited hepatitis B, an adequate immune response leads to viral elimination. Chronic HBV carriers probably suffer from an imbalanced immune response, insufficient to clear the virus but sufficient to cause tissue damage. These patients may be partially tolerant to viral antigens, e.g. HBsAg or HBcAg on the T-cell level [101]. Breaking a tolerance could exacerbate the disease as well as lead to viral clearance. This effect has been described in patients successfully treated with interferon.

In a pilot study, 10 woodchucks with naturally acquired chronic WHV infection were immunized either with WHcAg or HBcAg to test whether stimulation of the immune system against core antigens could break tolerance and induce virus clearance (table 2) [M. Roggendorf et al., unpubl. results]. One of 6 woodchucks immunized with WHcAg eliminated virus after four immunizations with WHcAg (fig. 4a). Sera were negative for WHV DNA from day 66 after the first immunization. In all other woodchucks, serum levels of WHV DNA and WHsAg did not change (fig. 4b). There was no increase of anti-WHc. The 4 woodchucks immunized with HBcAg developed anti-HBc and anti-HBe antibodies. However, none of the animals lost WHV. The anti-HBc response from WHV-chronic woodchucks and WHV-negative woodchucks was similar. However, the anti-HBe titers remained low in chronic carriers whereas the WHV-negative woodchucks developed titers up to 10^{-5}. The reason for this difference could be the partial tolerance to T-cell epitopes necessary for efficient anti-HBe production or cross-reaction between circulating WHeAg and anti-HBe in the chronically infected woodchucks. The one animal which lost WHV after immunization with WHcAg proteins had a low titer of WHV DNA prior to immunization than did other animals that did not eliminate the virus. This finding may be an indication that, due to the high replication level of WHV DNA, the stimulation of the immune system by core proteins would be sufficient to eliminate the virus. To clarify this, further experiments will be necessary, using an antiviral chemotherapy to reduce the viral load prior to immunization with core proteins. This approach of therapeutic vaccination is currently being investigated in human patients with chronic hepatitis B.

Table 2. Summary of therapeutic vaccination of WHV carrier woodchucks with recombinant core protein of WHV/HBV and WHsAg

Vaccine	Animals	WHV clearance	Persistent viremia
WHV core	6	1	5
HBV core	4	0	4
WHsAg	4	0	4
No vaccine	30[1]	0	30

[1] 25 historical controls (observation period 3 years) and 5 controls examined in parallel to vaccination with WHsAg.

Recently it has been shown that peptides that are major T-cell epitopes (aa 18–27) can elicit a CTL response in man as has been shown for other viruses [88]. Preclinical studies are currently being undertaken to immunize chronic HBV carriers, to test whether a sufficient CTL immune response to the core protein can be induced and virus elimination achieved [F. Chisari, pers. commun.].

A therapeutic vaccination approach has been used to cure mice with a persistent paramyxovirus infection (simian virus 5, SV5). Randall and Young [102] immunized mice with a solid matrix antibody-antigen (SMAA) complex which induced CD8+ effector cells. In a preliminary report, Wen et al. [103] succeeded in eliminating DHBV

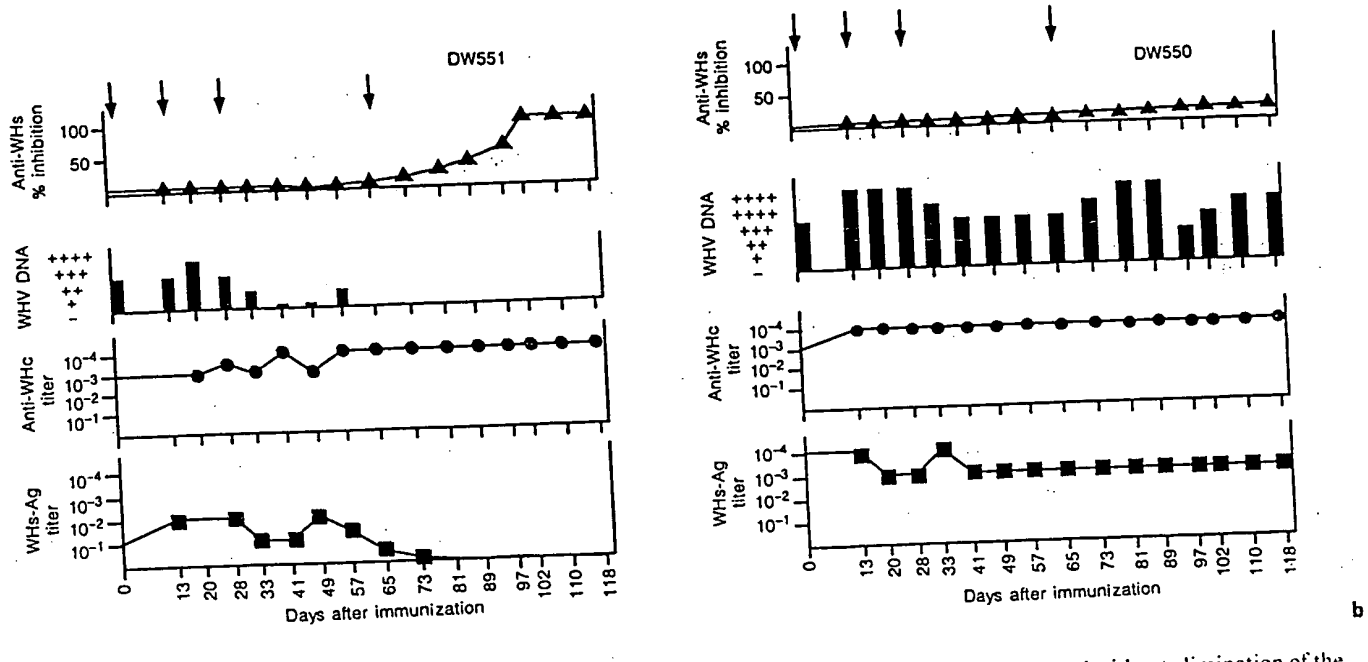

Fig. 4. Therapeutic vaccination of a chronic WHV carriers with core antigen with and without elimination of the virus: **a** woodchuck DW551: WHV elimination; **b** woodchuck DW550: WHV persistence.

in ducks with persistent DHBV infections using an SMAA complex containing either S-antigen or core antigen from DHBV.

In a small study, chronic WHV carrier woodchucks were immunized with WHsAg vaccine purified from plasma in CFA in an approach similar to that described by Pol et al. [99] (table 2). All woodchucks developed a transient anti-WHs response, but did not eliminate the virus [S. Hervas-Stubbs et al., unpubl. results]. The inability to eliminate WHV despite the presence of anti-WHs might be the high viral load and a low antibody titer. Indeed, the anti-WHs titer disappeared 3–4 weeks after the last immunization.

Limitations of the Woodchuck Model for Vaccination Studies

One major obstacle of the woodchuck model is that to date no inbred laboratory animals are available. Studies of model human immune responses at the cellular level will require phenotypic markers for T-cell subsets in the woodchuck. Research is currently in progress to define lymphocyte subsets in the woodchuck. Another technical limitation results from the fact that although woodchuck PBMC proliferate in response to various mitogens including the core protein, ^{3}H-thymidine incorporation is generally poor [57]. However, recent studies on stimulation of woodchuck PBMC with core protein of WHV show a significant incorporation of bromodeoxyuridine (BrdU) indicating lymphocyte proliferation [S. Menne et al., unpubl. data].

The Woodchuck as a Model for Evaluation of Antiviral Drugs

Prior to discovery of WHV, potential drugs against HBV were tested in vitro for their ability to inhibit the virion-associated DNA polymerase activity or in patients for the capacity to reduce serum levels of virus-associated DNA polymerase or of HBsAg (table 3). The first animal model of HBV infection, chimpanzees, were rarely used in such trials because of animal protection and high costs. WHV infection of woodchucks is a model in which the virus and associated disease have been relatively well characterized. Yet few in vivo studies of antiviral agents using woodchucks have been published [104–111]. The woodchuck model has the advantage of employing the virus most closely related to HBV, but the disadvantage of being relatively inaccessible or expensive for most laboratories. In contrast, the equally well-characterized DHBV-duck model is available around the world and has consequently been used more often to test potential antiviral substances. Drawbacks to the DHBV-duck system are that DHBV is much less similar to HBV than WHV and

Table 3. Antiviral drugs tested in WHV chronic carrier woodchucks

Drug	Effect on WHV replication (WHV DNA level in the serum)	Reference
Trisodium phosphonophosphate (foscarnet)	no effect	103
Adenine arabinoside monophosphate (ARA-AMP)	transient decrease	104
2′-Fluoro-5-ethyl-β-*D*-arabinofuranosyl-uracil (FEAU)[1]	transient decrease[1]	104, 107
Thymosin-α_1	transient decrease	107
Erythromycin-9-(0-methyl)oxine (EMO)	no effect	107
L-HSA conjugated		
ARA-AMP	transient decrease	110
Acyclovir	transient decrease	110
2′,3′-Dideoxycytidine (DDC)	transient decrease, no effect in animals with very high levels of viremia	111
DDC-MP	transient decrease, no effect in animals with very high levels of viremia	111
F-5-methCTP	transient decrease	105
cis-5-Fluoro-oxathiolan-cytosine	transient decrease	J. Cullen, pers. commun.

[1] Several animals died after long-term treatment.

GSHV and that avian metabolism and pharmacokinetics of the tested drugs may more significantly differ from those of mammals.

Trisodium phosphonophosphate (foscarnet) has been administered to WHV-infected woodchucks without benefit [103]. In general, nucleoside analogues produce only a transient depression in the levels of circulating virions, which return to pretreatment levels after the cessation of drug administration. This was shown with ARA-AMP (adenine arabinoside monophosphate), which is toxic in humans, as a model system for the testing of antiviral compounds in the woodchuck [107]. Treatment with FEAU (2′-fluoro-5-ethyl-β-*D*-arabinofuranosyl-uracil) leads to a transient decrease in serum WHV DNA after a 10-day treatment. A longer therapeutic regimen reduced effectively not only the serum WHV DNA but also the RNA transcripts in the liver. But within 4–14 days after the end of a long-term treatment, all of the treated animals died. No specific cause of death was identified [104, 107]. EMO (erythromycin-9-(O-methyl)oxine) has been reported to reduce the serum levels of DHBV in Pekin ducks [108]. EMO treatment of woodchucks did not reduce the levels of serum WHV DNA or WHsAg, the level of WHV replication, or the level of WHV transcription in hepatocytes [107]. The treatment of WHV chronic carrier woodchucks with thymosin-α_1 leads to a significant decrease in the levels of viremia. In the follow-up period, however, the viremia returned to pretreatment levels in all animals [109].

Lactosaminated human serum albumin (L-HSA), which might function as a selective drug vector for hepatocytes in vivo, is taken up specifically by the asialoglycoprotein receptor of hepatocytes. In the woodchuck model, Ponzetto et al. [110] showed that the conjugated drug is stable in serum, that the conjugate retains the capacity of L-HSA to bind to hepatocyte receptors and that the drug appears to be released in a pharmacologically active form in the target organ, based on the inhibition of viremia. The antiviral substances used for conjugation were ARA-AMP, acyclovir and 2′,3′-dideoxycytidine and its monophosphate [111]. The antiviral effects were transient.

The woodchuck model with a complete virus life cycle offers the advantage to test drug delivery methods, study pharmacokinetics, drug toxicity, and immune modulators. The in vivo model allows testing of drugs that affect WHV in all stages during its life cycle.

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